AWARD NUMBER: W81XWH-12-1-0474

TITLE: "MICRORNAS TO PATHWAYS IN PROSTATE CANCER PROGRESSION"

PRINCIPAL INVESTIGATOR: DR. ROBERT BLELLOCH, MD, PHD.

CONTRACTING ORGANIZATION: THE UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
San Francisco, CA 94103

REPORT DATE: October 2014

TYPE OF REPORT: ANNUAL

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	ANNUAL	30th Sept,2013 – 29th Sept,2014

4. TITLE AND SUBTITLE
MicroRNAs to Pathways in Prostate Cancer Progression

5a. CONTRACT NUMBER
–

5b. GRANT NUMBER
W81XWH-12-1-0474

5c. PROGRAM ELEMENT NUMBER
–

6. AUTHOR(S)
Robert Blelloch, MD, PhD.

E-Mail: BlellochR@stemcell.ucsf.edu

5d. PROJECT NUMBER
–

5e. TASK NUMBER
–

5f. WORK UNIT NUMBER
–

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of California, San Francisco
1855 Folsom Street, Ste. 425
San Francisco, CA-94103

8. PERFORMING ORGANIZATION REPORT NUMBER
–

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)
–

11. SPONSOR/MONITOR'S REPORT NUMBER(S)-

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The purpose of this Idea Development Award is to understand the molecular basis of early events in prostate cancer progression. In particular the proposal focuses on a class of non-coding RNAs called microRNAs that function to suppress large networks of genes during cell fate transitions. The proposal was based on preliminary data showing that in absence of all microRNAs, prostate tumors associated with PTEN loss fail to progress. The goal here is to determine the microRNAs and downstream-regulated pathways responsible for this striking block. In the past year we have isolated RNA from YFP-labeled prostates of the four genetic backgrounds (wild type, Dgcr8 knockout, PTEN knockout, and PTEN/Dgcr8 double knockout) and performed mRNA array analysis. Bioinformatic analysis of this data showed few changes likely secondary to poor correlation between reporter and loop out of PTEN and/or Dgcr8. We are currently testing alternative approaches for isolating mutant cells and performing miRNA and mRNA measurements with minimal RNA input to ensure improved homogeneity of cells evaluated.

15. SUBJECT TERMS nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	8	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

PROGRESS REPORT:

1. Introduction

The overall goal of this funded proposal is to dissect the molecular mechanisms of early tumor progression in the prostate. The proposed aims are based on preliminary findings showing that the loss of a specific class of regulatory molecules called microRNAs blocks progression from early hyperplasia to dysplasia in a PTEN knockout model of the disease. The aims themselves are to uncover the underlying microRNAs (aim 1) and microRNA regulated pathways (aim 2) that are normally responsible for progression. Knowing the miRNAs and downstream pathways that cooperate with PTEN loss (the most commonly deleted gene in prostate cancer) has the potential to improve the evaluation of the risk of early progression. The ability to distinguish and treat tumors that are likely to progress will have a broad impact on the disease as it would have the potential to diminish the overdiagnosis and overtreatment of early disease.

The goals for year two as outlined in the statement of work were to:

1) Profile RNA samples for miRNA and messenger RNA species (year one months 11-13)
2) Bioinformatics analysis and validation (month 14-16)
3) Building constructs (months 17-24)
4) Introduction of constructs into mice (months 22-30)

2. Keywords

Prostate Cancer, MicroRNAs, Post-transcriptional Regulation , Progression, Senescence, Basal and Luminal Cells, Transformation.

3. Accomplishments

Summary of Key Accomplishments

1) Development of mouse model that allows dissection of microRNA function during early stages of tumor development.
2) Development of methods to isolate and evaluate transformed cells from the otherwise heterogeneous cellular background of the prostate.
3) Preparation of high quality RNA from small numbers of cells allowing for future measurements of changes in mRNA and small RNA levels between different mutant backgrounds.
4) Improved sequencing strategies for measuring miRNAs from very small amounts of input RNA.

Our progress toward the aims of this proposal is as follows:

We have now evaluated earlier timepoints, identifying a suppression of progression by Dgcr8 loss even earlier than we previously suspected (i.e. at 12 weeks).

We performed array profiling for messenger RNAs from YFP-sorted cells from four mice of each genotype. YFP expression was driven by the probasin-cre transgene and therefore should label the cells that also had cre-mediated deletion of Pten and/or DGCR8. Unfortunately the arrays were not informative. As shown in Figure 1a, the samples clustered based on PTEN. However loss of DGCR8 did not change the profile in a significant way. There were no significant expression changes between PTEN knockout prostates and PTEN/Dgcr8 double knockout prostates. There are a number of explanations for this including that YFP is not a robust marker for cells in the mouse that have complete loop out of all floxed alleles.

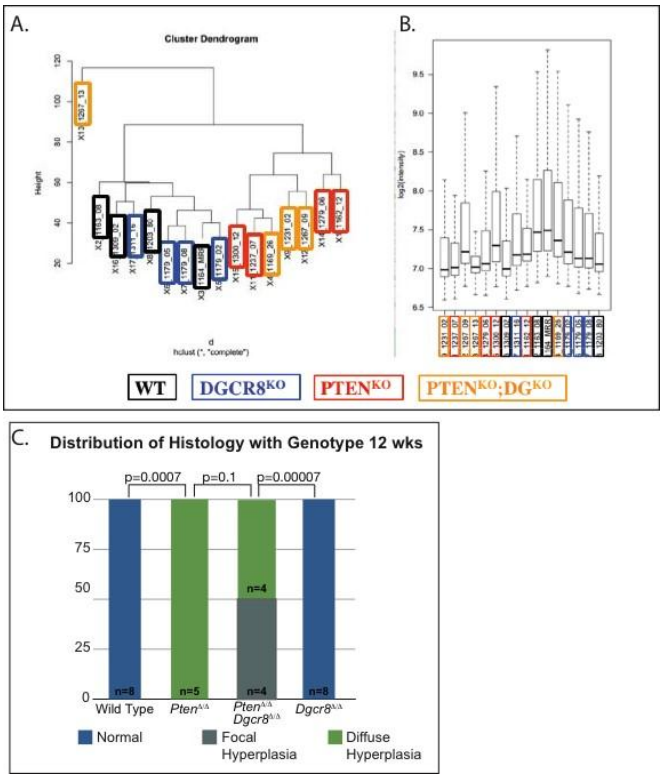

Figure 1: Illumina microarray analysis of expression differences in four prostates of each genotype WT (black), Dgcr8^{KO} (Blue), Pten^{KO} (Red) and Pten/Dgcr8 double KO (Orange). A.) clustering of expression divides the samples into two arms based on Pten expression. B.) Box plot of mRNA expression levels of each sample shows that the expression levels in general were lower than expected and the dynamic range was too tight and variable. 2.) Histology of 12 week aged prostates from the different genotypes. PTEN^{KO} prostates were uniformly hyperplastic while half of the double knockouts showed focal hyperplasia.

We performed the profiling on 12 week old prostates. Histological analysis and immunophenotyping was performed on matching tissues from the same prostates. Immune staining for YFP expression was not uniform at this time point in all prostates.. However in the Pten^{KO} prostates, glands were uniformly hyperplastic suggesting that that PTEN was lost in most cells and thus there being a disconnect between YFP and PTEN losss. In the Pten^{KO}/Dgcr8^{KO} prostates showed some focal hyperplasia, which could also be explained by incomplete loop out of all alleles across cells. Another issue with the array data was that the dynamic range in signal was low as shown in Figure 1b, likely secondary to the extensive amplification required to get signal.

4.) Impact

Our studies to date demonstrate that there is a requirement for microRNAs for the histological progression of PTEN^{KO}-mediated prostate cancer. As early as 12 weeks there is a reduction in hyperplasia in the PTEN/Dgcr8 double knockouts compared to PTEN knock-out and any further progression is entirely blocked. The block is associated with cell senescence thereby identifying a central role for miRNAs in promoting prostate cancer progression by overcoming this important barrier. As such it provides a targetable node in prostate cancer for treatment. We are now

searching for specific miRNAs that underlie this regulation with the ultimate goal of testing how delivery of the miRNAs and inhibitors to miRNAs can be used to manipulate this node. Such knowledge could lead to future drug treatments.

5.) Changes/Problems

As described under accomplishments, we have run into a number of problems with profiling the tumors including tissue heterogeneity, imperfect activation of our reporter relative to loop out of other alleles, and small amount of RNA we are able to purify from the tumors. We are working on a number of solutions as described below. We also suffered a setback when the mice stopped breeding and required rescue by out-crossing to the wild-type line. It took several months to rebuild homozygous lines.

Solutions to problems with reporter lines and limited RNA yield.

A.) Use whole prostates as input for profiling. A recent study suggests that expression profiling of whole prostates may reveal global differences in prostate expression[1]. We have revised tissue digestion methods that result in digestion with better cell viability, which will lead to higher quantity and quality of RNA. This strategy avoids the issue of trying to get pure tissues using the YFP marker in the different genetic backgrounds.

B.) FACS sort based on surface markers for prostate epithelial cells instead of using the YFP expression as a marker. The approach described in alternate plan A suffers from the heterogeneity of the whole tissue. Therefore, we are also testing sorting basal and luminal populations, from stromal cells. Our colleague, Davide Ruggero, has recently succeeded in using the markers CD49f and Sca-1 to do exactly this in the PTEN knockout model. He will help us set it up in our model. As probasin-cre is broadly active in the epithelium, this approach should give us mostly looped out cells.

C.) Develop new profiling methods that can use very low RNA input: In the realm of miRNA profiling, we have been working on novel methods to build libraries from very little input RNA (less than or equal to 1 ng) (Figure 2). This method will allow us to measure the miRNAs, even if we are unable to improve our yield of RNA material from the sorted prostates.

D) Measure miRNAs in human prostate tissue and serum. Reviewers of this grant requested that we use human materials along with our mouse work. We have been hesitant to use cell lines as they are even more distant from the human disease than the mouse models. However, with our improved library techniques, we have been able to sequence miRNAs from small prostate biopsies and even serum. We have successfully isolated miRNAs from serum samples and used deep sequencing to measure relative miRNA levels (Figure 3). This provides an unbiased approach for screening for miRNAs relevant to human prostate cancer progression. We then propose to use the same bioinformatics approach as previously proposed to determine mRNA targets of these miRNAs. We can then utilize the mouse model to test the functionality of the miRNAs and their impact on mRNA expression pathways

on prostate cancer development and progression as described in part b of both aims.

6.) Products

We expect to submit our first manuscript this year describing the phenotype associated with microRNA loss in the Pten null prostate without profiling of mRNAs and miRNAs.

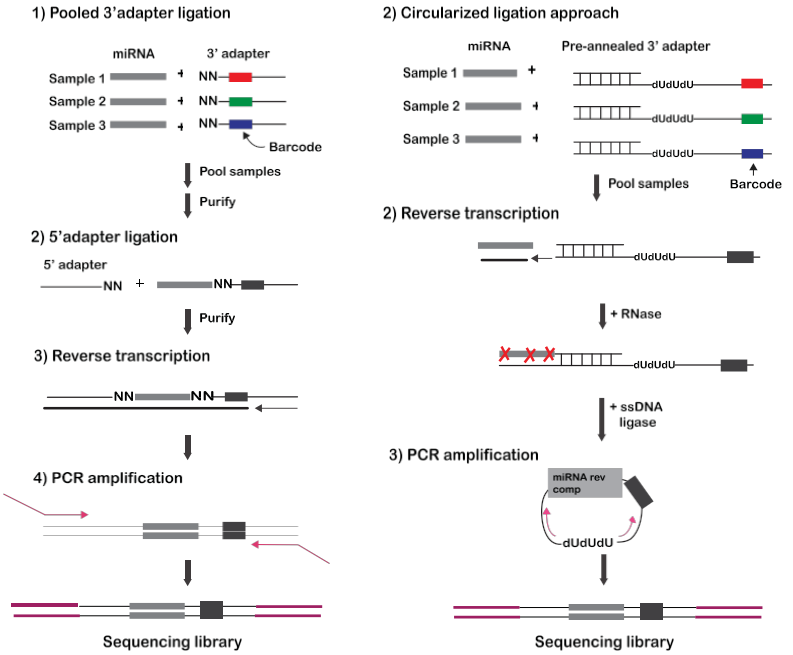

Figure 2: Improved methods to build small RNA libraries from minimal input RNA for deep sequencing.

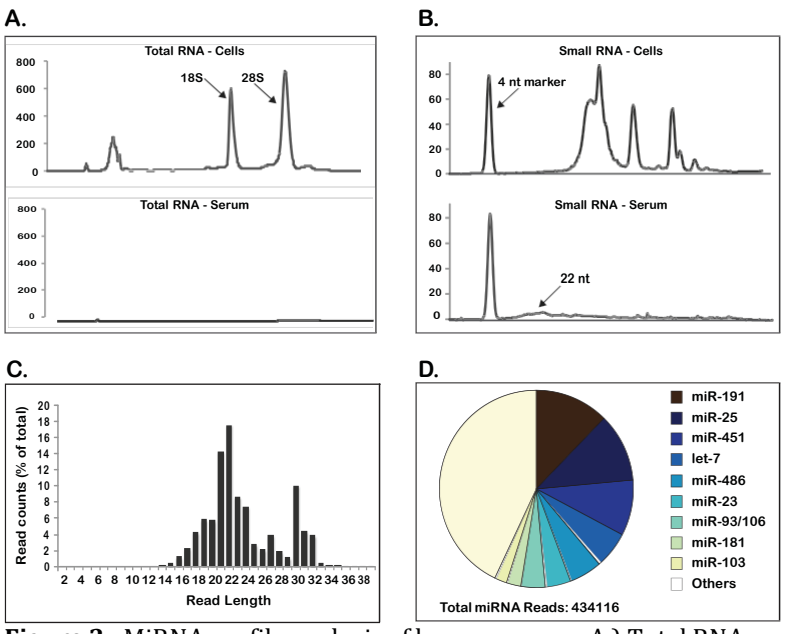

Figure 3: MiRNA profile analysis of human serum. A.) Total RNA purified from cells is predominantly ribosomal. B.) However in serum the 22 nt miRNA species is the dominant form. C.) Sequencing of a serum library produces read lengths of the expected size. D.) Over 500 miRNA species are found in blood, although top 9 microRNAs make up over 50% of the pool.

Reference

1. Zhang B, Chen H, Zhang L, Dakhova O, Zhang Y, et al. (2014) A dosage-dependent pleiotropic role of Dicer in prostate cancer growth and metastasis. Oncogene 33: 3099–3108. doi:10.1038/onc.2013.281.